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(54) Title: STRESS PROTEINS AND USES THEREFOR

(57) Abstract

Stress proteins and their use to immunize an individual against a nonviral infection or to induce immune tolerance in an
individual, as well as a method of immunizing an individual by administering a selected stress protein and a method of inducing
immune tolerance in an individual by administering a selected stress protein.

AM

STRESS PROTEINS AND USES THEREFOR

Description

Background of the Invention

Although the function of stress proteins is not
05   entirely clear, it appears that some participate in
assembly and structural stabilization of certain cellular
and viral proteins, and their presence at high
concentrations may have an additional stabilizing effect
during exposure to adverse conditions. Neidhardt, F.C.
10   and R.A. VanBogelen, In: Escherichia coli and Salmonella
typhimurium, Cellular and Molecular Biology, (eds.
Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B.
Schaechter, M. and Umbarger, H.E. (Am. Soc. Microbiol.,
Washington, D.C.), pp. 1334-1345 (1987); Pelham, H.R.B.
15   Cell, 46:959-961 (1986); Takano, T. and T. Kakefuda,
Nature, 239:34-37 (1972); Georgopoulos, C. et al., New
Biology, 239:38-41 (1972). Phagocytic host cells produce
a hostile environment for foreign organisms, and the
ability to produce stress proteins has been implicated in
20   the survival of bacterial pathogens within macrophages
Christman, M.F. et al., Cell, 41:753-762 (1985).

Mycobacterium (M.) tuberculosis and Mycobacterium
(M.) leprae are the etiologic agents of tuberculosis and
leprosy, respectively. These diseases afflict 20-30
25   million people and continue to present a significant
global health problem. Joint International Union Against
Tuberculosis and World Health Organization Study Group,
Tubercle, 63:157-169 (1982); Bloom, B. and T. Godal, Rev.
Infect Dis. 5:765-780 (1983). To develop more effective

-2-

tools for the diagnosis and prevention of these diseases,
it is important to understand the immune response to
infection by mycobacterial pathogens.

The antibody and T-cell responses to infection or
05 inoculation with killed mycobacteria have been studied in
humans and in animals. Human patients with tuberculosis
or leprosy produce serum antibodies directed against at
least 12 mycobacterial proteins. Some of these proteins
are also recognized by well-characterized murine
10 monoclonal antibodies. Mice immunized with mycobacterial
lysates produce antibodies that are directed
predominantly to six M. tuberculosis and six M. leprae
protein antigens. Engers, H.D., Infect. Immun.,
48:603-605 (1985); Engers, H.D., Infect. Immun.,
15 51:718-720 (1986). Genes encoding these 12 mycobacterial
antigens have been cloned, and recombinant proteins
produced from these clones have been used to investigate
the human T-lymphocyte response to mycobacterial
infection. Husson, R.N. and R.A. Young, Proc. Natl.
20 Acad. Sci., USA, 84:1679-1683 (1987); Young, R.A. et al.,
Nature, 316: 450-452 (1985); Britton, W.J. et al., Lepr.
Rev., 57, Suppl. 2, 67-75 (1986).

Protection against mycobacterial disease involves
cell-mediated immunity. Joint International Union
25 Against Tuberculosis and World Health Organization Study
Group, Tubercle, 63:157-169 (1982); Hahn, H. and S.H.E.
Kaufman, Rev. Infect. Dis., 3:1221-1250 (1981). T
lymphocytes cloned from patients or from volunteers
immunized with killed mycobacteria have been tested for
30 their ability to recognize the recombinant mycobacterial
proteins. Lymphocyte-proliferation assays demonstrate

-3-

that most of the antigens identified with monoclonal
antibodies are involved in the T-cell response to
mycobacterial infection or vaccination in mice and in
humans. Limiting dilution analysis indicates that 20% of
05   the mycobacterial-reactive $CD4^+$ T lymphocytes in mice
immunized with M. tuberculosis recognize a single
protein, the 65-kDa antigen. Kaufman, S.H.E. et al., Eur
J. Immunol., 17:351-357 (1987).

Summary of the Invention
10   The present invention relates to stress proteins and
methods of modulating an individual's immune response,
either to a pathogen or to his or her own cells, such as
occurs in autoimmune diseases. In particular, it relates
to the use of such stress proteins as a "vaccine" in
15   immune prophylaxis therapy, which results in an induction
or enhancement of immune response to a selected pathogen
and as an immunotherapeutic agent in treatment of
autoimmune diseases, which results in a decrease of an
individual's response to his or her own cells. In immune
20   prophylaxis, stress proteins are administered to prevent
or reduce the effects in an individual of a pathogen,
which can be any virus, microorganism or other organism
or substance (e.g., a toxin or toxoid) which causes
disease. In preventing or reducing adverse effects of
25   nonviral pathogens (e.g., bacteria, mycobacterial)
according to the method of the present invention, an
individual's immune response to the nonviral pathogen's
stress protein(s) is induced or enhanced through the
administration of a vaccine which includes the pathogen's
30   stress protein(s) and, generally, an adjuvant.

-4-

Preventing or reducing adverse effects of viral
pathogens, as well as preventing cell transformation or
reducing the extent to which it occurs, according to the
present method, is effected by transiently enhancing an
05   individual's immune surveillance system. In this
instance, the causative pathogens (i.e., virus;
transforming agent) do not have stress proteins of their
own. Enhancement of immune response can be effected by
modulating the immune cells by stimulation with a
10   nonviral stress protein (e.g., a bacterial stress
protein) or modulating the individual's stress response
by any means (e.g., local application of heat).

In immune therapy, such as is used in treating
autoimmune diseases, stress proteins known to be involved
15   in the autoimmune response are administered to turn down
an individual's immune response by tolerizing the
individual to the stress proteins. Alternatively, the
immune response to stress protein, which is known to
occur in autoimmune disease, is reduced by interfering
20   with the ability of immune cells which respond to stress
proteins to do so.

A selected stress protein of the present invention
can be administered to an individual, according to the
method of the present invention, and result in an immune
25   response which provides protection against subsequent
infection by a nonviral organism (e.g., bacteria,
mycobacteria, other infectious agents which produce
stress proteins). Alternatively, a selected stress
protein can be administered to an individual, generally
30   over time, to induce immune tolerance against the
selected stress protein. For example, a selected stress

-5-

protein can be administered in multiple doses over time
in order to induce immune-tolerance against an autoimmune
disease such as rheumatoid arthritis.

Brief Description of the Drawings
05   Figure 1 is a graphic representation of the
homologies between mycobacterial antigens and known
stress proteins. Figure 1A is a representation of
sequence similarity between portions of the M. tuberculo-
sis 71-kDa antigen (residues 1-204; TB 71kDa) and the E.
10   coli DnaK protein (residues 430-469). Figure 1B is a
representation of sequence similarity between portions of
the M. tuberculosis 65-kDa antigen (residues 1-540; TB
65kDa) and the E. coli GroEL protein (residues 1-547).
             Figure 2 is a comparison of the amino acid sequence
15   of the human P1 protein (573 residues) and the amino acid
sequence of the groEL protein (547 residues).
             Figure 3 is a comparison of the amino acid sequence
of the human P1 protein (573 residues), which is a
homolog of groEL protein, and the amino acid sequence of
20   the 65kDa M. leprae protein (540 residues).
             Figure 4 is a comparison of the amino acid sequence
of the human P1 protein (573 residues), which is a
homolog of the groEL protein, and the amino acid sequence
of the 65kDa M. tuberculosis protein (540 residues).

25   Detailed Description of the Invention
             The present invention is based on the observation
that stress proteins are among the major antigens
available for presentation to T lymphocytes and may be
common immune targets in a broad spectrum of infectious

diseases. Immune responses to stress proteins are
involved in immune surveillance by the body and a variety
of different T cell types has been shown to recognize
highly conserved stress protein determinants. Several
05 observations, described below, suggest a model of immune
surveillance in which self-reactive T cells provide a
first line of defense against infection or other invasion
by pathogens and against cell transformation by
recognizing and helping to eliminate stressed autologous
10 cells, as well as cells infected with intracellular
bacteria. Without wishing to be bound by this model, it
is presented as one means by which it is possible to
explain why prokaryotic and eukaryotic cells respond to a
variety of potentially damaging stimuli, such as elevated
15 temperature, by increasing the synthesis of a family of
proteins, referred to as stress proteins, which are among
the most highly conserved and abundant proteins found in
nature.

    Investigation of antigens involved in the immune
20 response to the tuberculosis and leprosy bacilli (M.
tuberculosis and M. leprae) initially led to the
observation that a variety of stress proteins are among
the major targets of the immune response, as is described
at greater length below.

25   Further assessment has demonstrated that stress
proteins may be common immune targets in a broad spectrum
of infectious diseases. Sequence analysis has revealed
70-kDa heat shock protein homologues among major antigens
of the protozoan parasites Plasmodium falciparum (Bianco,
30 A.E. et al., Proc. Natl. Acad. Sci., USA, 83:8713-8717
(1986)) and Schistosoma mansoni (Hedstrom, R. et al., J.

-7-

Exp. Med., 165:1430-1435 (1987)) and the malarial
parasite Brugia malayi (Selkirk, M.E. et al., J. Cell
Biochem., 12D:290 (1988)). Similarly, homologues of
GroEL have been found among antigens involved in the
05   immune response to Salmonella typhimurium and Coxiella.
Vodkin, M.H. and J.C. Williams, J. Bacteriol, 170:1227
(1988). The presence of stress proteins among major
immune targets in a variety of human pathogens is support
for the idea that the stress response may be a general
10   component of infection and that stress proteins should be
considered among candidates for subunit vaccines. All
organisms respond to heat by inducing synthesis of heat
shock proteins (hsps), which are a group of proteins.
This response is the most highly conserved genetic system
15   known and has been shown to occur in every organism,
including microorganisms, plants and animals,
investigated to date. Many of the characteristics of the
response are common to all organisms and the hsps are
among the most highly conserved proteins known. For
20   example, hsp90 family and hsp70 family proteins are
present in widely diverse organisms. The proteins in
each family--even in such diverse organisms--show
approximately 50% identity at the amino acid level and at
the nonidentical residues, exhibit many similarities.
25   Several of the proteins induced by heat are also induced
by a variety of other stresses. The hsps or a closely
related/similar protein are present in all organisms at
normal temperatures and have been shown to have key
functions in normal cell metabolism. Lindquist, S. and
30   E.A. Craig, Ann. Rev. Genet., 22:631-677 (1988). Because
the stress response is common to prokaryotes and

-8-

eukaryotes and stress proteins are among the most highly
conserved in sequence, it is reasonable to expect that an
antigen from one pathogen could immunize against another
pathogen. Exposure to foreign stress proteins early in
05 life might, in fact, induce a degree of immunity to a
variety of infectious agents. If so, this could provide
an explanation for the observation that, for many
pathogens, only a fraction of infected individuals
actually acquire clinical disease.
10   The following is a description of the relationship
which has been observed between stress proteins and the
immune response to mycobacterial infection; of the
observation and supporting information that stress
proteins are immune targets in many non-viral infections;
15 of recognition of the fact that immune responses to
conserved stress protein determinants may play an
important role in autoimmune pathology in rheumatoid
arthritis, as well as in adjuvant arthritis; and of the
role of stress proteins in immune surveillance, as well
20 as a model proposed for immune surveillance in which
self-reactive T cells provide a first line of defense
against infection and cell transformation.

Mycobacterial Stress Proteins are Targets of the Immune
Response
25   An intriguing relationship between stress proteins
and the immune response to mycobacterial infection has
been observed. A more detailed examination of stress
protein determinants and immune response mechanisms is
essential to understanding the relationships among stress
30 proteins, infection, and immunity.

        In view of the involvement of proteins of M.
tuberculosis and M. leprae in humoral and cell-mediated
immune responses and to establish the functions of the
these proteins in the mycobacterial cell, the DNA
05 encoding several of the M. tuberculosis and M. leprae
antigens have been sequenced. It has been demonstrated,
as a result, that many of these mycobacterial protein
antigens exhibit striking sequence similarity to known
stress-induced proteins.   Three of the M. leprae and
10 two of the M. tuberculosis protein antigens studied have
been shown to exhibit striking sequence similarity to
known stress proteins. For reasons discussed in the
Exemplification, it is concluded that two of the M.
leprae and two of the M. tuberculosis antigens are
15 homologues of the E. coli DnaK and GroEL proteins.
        In experimental mice, immunization with
mycobacterial lysates elicits antibody responses to at
least six M. tuberculosis protein antigens and a similar
number of M. leprae protein antigens. Monoclonal anti-
20 bodies specific for these proteins have been used to
isolate clones from λgt11 DNA expression libraries of M.
tuberculosis and M. leprae. The sequence of the DNA
clones revealed that mycobacterial hsp70 (alias 70 kDa
antigen) and hsp60 (alias 65 kDa antigen, groEL) were the
25 major targets of the murine antibody response to both M.
tuberculosis and M. leprae. Two additional hsp's, an 18
kDa member of the small hsp family and a 12 kDa homologue
of groES, were found among the M. leprae and M.
tuberculosis antigens. Young, D.B., et al., Proc. Natl.
30 Acad. Sci., USA, 85:4267-4270 (1988); Shinnick, T.M., et
al., Nuc. Acids Res., 17:1254 (1989).

-10-

The mycobacterial stress proteins are among the immunodominant targets of both murine antibody and T cell responses. In one study which summarized results obtained from 10 laboratories, a collection of 24 murine

05 monoclonal antibodies recognized 6 M. leprae proteins; 7 of these antibodies are directed against 6 different determinants in the M. leprae hsp60. Engers, H.D., et al., Infect. Immun., 48:603-605 (1985); Mehra, V., et al., Proc. Natl. Acad. Sci. USA, 83:7013-7017 (1986).

10 In a similar study, 3 of 33 monoclonal antibodies raised against M. tuberculosis recognized the M. tuberculosis hsp60 protein. Engers, H.D., et al., Infect. Immun., 51:718-720 (1986). Finally, limiting dilution analysis indicates that 20% of the mycobacterial-reactive $CD4^+$ T

15 lymphocytes in mice immunized with M. tuberculosis recognize this antigen. Kaufmann, S.H., et al., Eur. J. Immunol., 17:351-357 (1987).

Although a rigorous quantitative analysis of the human immune response to mycobacterial stress proteins

20 has not yet been reported, mycobacterial stress proteins are recognized by human antibodies and T lymphocytes and the evidence suggests that these proteins are among the major targets of the human cell-mediated immune response. Emmrich, F., et al., J. Exp. Med., 163:1024-1029 (1985);

25 Mustafa, A.S., et al., Nature (London), 319:63-66 (1986); Oftung, F., et al., J. Immunol., 138:927-931 (1987); Lamb, J.R., et al., EMBO J., 6:1245-1249 (1987). T lymphocytes from patients with mycobacterial infection or from volunteers immunized with mycobacteria have been

30 cloned and tested for their ability to recognize the mycobacterial stress proteins. In each of these studies,

-11-

some fraction of the human T cell clones were shown to
recognize one or more of the mycobacterial stress
proteins.

Stress Proteins are Immune Targets in Non-viral
05 Infections
        The observation that stress proteins are important
targets of the immune response to mycobacterial infection
and the knowledge that the major stress proteins are
conserved and abundant in other organisms suggested that
10 stress proteins are likely to be immune targets in many
non-viral infections. Indeed, that is now clearly the
case. Antigens from a wide variety of infectious agents
have been identified as members of stress protein
families. The major stress protein antigen recognized by
15 antibodies in bacterial infections is hsp60. "Common
antigen", an immunodominant protein antigen long known to
be shared by most bacterial species, turns out to be
hsp60. Shinnick, T.M., et al., Infect. Immun., 56:446
(1988); Thole, J.E.R., et al., Microbial Pathogenesis,
20 4:71-83 (1988). Stress proteins have also been
identified as immune targets in most major human parasite
infections. Bianco, A.E., et al., Proc. Natl. Acad.
Sci., USA, 83:8713 (1986); Nene, V., et al., Mol
Biochem. Parasitol., 21:179 (1986); Ardeshir, F., et al.,
25 EMBO J., 6:493 (1987); Hedstrom, R., et al., J. Exp.
Med., 165:1430 (1987); Selkirk, M.E., et al., J. Cell
Biochem., 12D:290 (1988); Engman, D.M., et al., J. Cell
Biochem., 12D: Supplement, 290 (1988); Smith, D.F., et
al., J. Cell Biochem., 12D:296 (1988). Antibodies to
30 hsp70 have been identified in the sera of patients

-12-

suffering from malaria, trypanosomiasis, leishmaniasis,
shistosomiasis and filariasis. Hsp90 is also a target of
antibodies in trypanosomiasis and a member of the small
hsp family is recognized in some patients with
05 shistosomiasis.

Stress Proteins and Autoimmune Processes

     Rheumatoid arthritis is characterized by a chronic
proliferative and inflammatory reaction in synovial
membranes which is thought to involve autoimmune
10 processes. Rat adjuvant arthritis resembles human
rheumatoid arthritis in many respects, and has been used
as an experimental animal model for human disease.
Pearson, C.M., Arthritis Rheum., 7:80-86 ((1964).
Adjuvant arthritis can be induced in rats with a single
15 intradermal injection of killed M. tuberculosis in
complete Freund's adjuvant. An autoimmune process
involving T lymphocytes appears to be responsible for the
generation of the disease. Holoshitz, J., et al.,
Science, 219:56-58 (1983). T cell lines isolated from
20 the draining lymph nodes of arthritic rats and propagated
in vitro by stimulation with M. tuberculosis-pulsed
syngeneic antigen presenting cells can cause a transient
form of the disease when transferred to irradiated rats.
Since care was taken in these experiments to exclude the
25 transfer of contaminating M. tuberculosis, this result
strongly suggests that the clinical effects of the
disease are a consequence of an autoimmune reaction in
which the autoantigen is shared with M. tuberculosis.
     The rat and M. tuberculosis antigens recognized by
30 the arthritogenic T cells have been sought for a number

-13-

of years.. A number of different proteins present in
synovial membranes have been proposed to be the
cross-reactive rat antigen, but were later discounted as
procedures for the purification of these proteins
05 improved. van Eden, W., et al., _Proc. Natl. Acad. Sci._,
USA, _82_:5117-5120 (1985); Holoshitz, J., et al., _Science_,
_219_:56-58 (1983). The _M. tuberculosis_ antigen recognized
by the arthritogenic T cells was recently shown to be a
65 kDa protein (van Eden, W., et al., _Nature_, _331_:171
10 (1988), which has now been shown to be hsp60 (see the
Exemplification). Using a combination of truncated
recombinant 65 kDa proteins and peptides, a nine amino
acid epitope of hsp60 has been identified as the minimum
stimulatory sequence for arthritogenic T cell clones in
15 proliferation assays. Now that it is clear that some
arthritogenic T cells recognize the mycobacterial hsp60,
it is quite possible that the rat autoantigen is also
hsp60.
      The results obtained in the adjuvant arthritis model
20 led investigators to determine whether T lymphocytes from
human rheumatoid arthritis patients also recognize
mycobacterial antigens. These investigators have found
not only that patients with rheumatoid arthritis have T
cells that recognize _M. tuberculosis_ antigens, but that
25 these T cells have diverse phenotypes. Substantial
proliferative responses to mycobacterial extracts are
observed with uncloned T cells (predominantly CD4^{+}) from
both synovial infiltrates and peripheral blood, although
responses are generally greater in synovial infiltrates.
30 Abrahamson, T.G., et al., _Scand. J. Immunol._, _7_:81-90
(1978); Holoshitz, J., et al., _Lancet ii_, 305-306 (1986).

-14-

Holoshitz et al. found that 4 of 5 T cell clones isolated
from human rheumatoid synovia which respond to M.
tuberculosis antigens were CD4⁻ CD8⁻ cells with γ/δ T
cell receptors. Holoshitz, J., et al., Nature,
05   339:226-229 (1989). This observation is interesting
because γ/δ T cells have yet to be assigned a role in
immunity. One of the γ/δ clones was tested for its
ability to respond to purified mycobacterial hsp60 and
was found to be positive in proliferation assays. Due to
10   the conserved nature of stress proteins, these T cells
have the potential for autoreactivity. Lamb and
coworkers have shown that polyclonal T cells from
synovial infiltrates recognize both mycobacterial hsp60
and hsp70. Lamb, J.R., et al., Intl. Immunol., in press
15   (1989). The population of T cells that recognize the
mycobacterial stress proteins were shown to respond to E.
coli hsp60 and hsp70 and, most interestingly, human hsp70
purified from heat shocked macrophages. Thus, immune
responses to conserved stress protein determinants,
20   perhaps initiated by bacterial infection (not necessarily
by mycobacteria), may play an important role in
autoimmune pathology in rheumatoid arthritis, as well as
in adjuvant arthritis.

Stress Proteins and Immune Surveillance
25   A variety of different T cell types has now been
shown to recognize highly conserved stress protein
determinants. The ability of cells to respond to stress
by increasing the levels of the highly conserved stress
proteins; the presence of T cells of diverse phenotypes
30   in healthy individuals that are capable of recognizing

-15-

self stress protein determinants; and observations that
stress responses are induced by viral infection and by
cell transformation, all suggest a model of immune
surveillance in which self-reactive T cells provide a
05   first line of defense against infection and
transformation by recognizing and helping to eliminate
stressed autologous cells, as well as cells infected with
intracellular bacteria. The pool of lymphocytes that
recognize conserved stress protein determinants might be
10   induced during establishment of natural microbial flora
on the skin and in the gut, and maintained by frequent
stimulation by bacteria and viruses as well as other
stressful stimuli encountered during a normal lifetime.
This model is attractive because it provides a way in
15   which the immune system could exploit the existence of
conserved epitopes in stress proteins to respond
immediately to antigenically diverse pathogens and
cellular changes, producing an initial defense that need
not await the development of immunity to novel antigens.
20   Stress protein induction occurs in eukaryotic cells
following infection by diverse viruses in vitro.
Collins, P.L., and Hightower, L.E.; J. Virol., 44:703-707
(1982); Nevins, J.R., Cell; 29:913-939 (1982); Garry,
R.F., et al., Virology, 129:391-332 (1988); Khandjian,
25   E.W. and Turler, H.; Mol. Cell Biol., 3:1-8 (1983);
LaThangue, N.B., et al., EMBO J., 3:267-277 (1984). CTL
that recognize these neo-antigens could limit the spread
of virus by killing infected cells, possibly before
substantial amounts of mature virus are assembled, and by
30   secreting the lymphokine γ-interferon. Pestka, S., in:
Methods Enzymol., Interferons, Part A., Vol. 79, Academic

-16-

Press, New York, pp.667 (1981). Evidence consistent with this idea is emerging. Koga et al., have shown that infection of primary murine macrophages with CMV rendered them susceptible as targets for MHCOI restricted CD8^{+} CTL specific for linear epitopes of M. tuberculosis hsp60. Koga, T., et al. Although the epitope recognized by these CTL on infected macrophages was not defined, it is tempting to speculate that a cross-reactivity with self hsp60 epitopes is being observed. Indeed, the same groups showed that a homologous hsp60 is constitutively present in macrophages and is upregulated by γ-interferon stimulation.

T cells capable of recognizing autologous cells stressed by transformation could help eliminate nascent tumour cells. Stress proteins appear to be produced at high levels in at least some transformed cells. Bensaude, O. and Morange, M., EMBO J., 2:173-177 (1983). An 86 kDA murine tumour antigen has been found to be homologous to representatives of the hsp90 family in yeast and Drosophila. Ullrich, S.J., Proc. Natl. Acad. Sci. USA, 83:3121-3125 (1986). Immunization of mice with the purified protein led to inhibition of tumor growth in 95% of experimental animals that had been seeded with cultured tumor cells. All of the protected mice had high titres of anti-hsp90 serum antibody which was able to precipitate murine hsp90 from lysates of heat shocked mouse embryo cells. Again, a role for autoreactive lymphocytes is implied.

The lymphocytes which recognize conserved stress protein determinants must be capable of discriminating between normal and stressed cells. Since many stress

-17-

proteins are constitutively expressed in normal cells,
although at lower levels than in stressed cells, the
potential for autoreactivity is ever-present. Normal
cells may escape destruction by expressing only
05   substimulatory levels of stress protein determinants on
their surfaces. In addition, stress proteins may only be
processed and presented during stress, and it may be
relevant that many stress proteins have altered
intracellular locations during stress. Finally, immune
10   regulatory networks may prevent activation of
autoreactive T cells under normal conditions. The
regulatory constraints required by this system might
occasionally break down, perhaps during stress caused by
bacterial or viral infections, leading to autoimmune
15   disease. Rhematoid arthritis may be such a disease.

Modulation of Immune Response

The precise relationship between stress proteins and
the host immune response to infection is as yet
undefined. When cells are subjected to a variety of
20   stresses, they respond by selectively increasing the
synthesis of a limited set of stress proteins. Some
stress proteins, including the products of dnaK and
groEL, are major constituents of the cell under normal
growth conditions and are induced to even higher levels
25   during stress. Lindquist, S., Annu. Rev. Biochem., 55:
1151-1191 (1986); Neidhardt, F.C. and R.A. VanBogelen, In
Escherichia coli and Salmonella Typhimurium, Cellular and
Molecular Biology, (eds. Neidhardt, F.C., Ingraham, J.L.
Low, K.B. Magasanik, B. Schaechter, M. and Umbarger,
30   H.E.) Am. Soc. Microbiol., Washington, D.C., pp. 1134-
1345 (1987). It has now been demonstrated that stress-

-18-

related proteins are targets of the immune response.
It is reasonable to expect that immunodominant antigens
would be found among such abundant proteins, as has been
shown to be the case.

05   Stress-induced proteins or their functional
equivalents, can be used to immunize an animal against a
nonviral infection or, alternatively, other selected
stress-induced proteins, or their functional equivalents,
can be used to induce immune tolerance in an animal.

10   According to the method of the present invention, it is
possible to modulate the immune response in an individual
by altering the individual's response to stress proteins.
In particular, it is possible to modulate an individual's
response to a pathogen (e.g., bacterium, mycobacterium,

15   virus, or other organism or agent, such as parasites,
toxins, toxoids, which causes disease or cell transforma-
tion); and to modulate an individual's autoimmune
response, such as occurs in some forms of arthritis.

          It is possible to use stress proteins, as described

20   herein, as a vaccine which, after administration to an
individual, produces or enhances an immune response in
that individual, providing protection against subsequent
infection. Because, as demonstrated, these are proteins
in which there are regions of highly conserved amino acid

25   sequences and such proteins have been shown to be
ubiquitous targets in mycobacterial, bacterial and other
infections, they can be used to elicit an equally
ubiquitous immune response.

          For example, in the case of a pathogen which

30   expresses stress proteins, such as nonviral pathogens,
two approaches to enhancing an individual's immune

-19-

response (and, thus, reducing the pathogen's effects) can
be used.

First, because the nonviral pathogen's stress
proteins are distinguishable from those of the host, it
05   is possible to induce an immunoprophylactic response
specific to the pathogen's stress proteins. This can be
carried out by administering a vaccine which includes all
or a portion (e.g., sufficient sequence to have the
desired stimulatory effect on immune response) of the
10   pathogen's stress protein or of another protein having an
amino acid sequence sufficiently similar to that of the
stress protein sequence to stimulate the immune response
to the pathogen. Alternatively, highly conserved stress
protein determinants, such as those shown to be
15   recognized by a variety of T cells, can be administered
as a type of "general" vaccine. In either case, the
immune response to the stress protein sequence will be
increased and effects of the nonviral pathogen will be
reduced (decreased, prevented or eliminated).
20   Second, it is also possible to induce or enhance the
immune surveillance system or response which is directed
to stressed host cells. This is described further in the
context of enhancing immune response in those instances
in which the pathogen (e.g., a virus, transforming agent)
25   does not have (express) its own stress proteins (i.e.,
stress proteins distinguishable from host stress
proteins).

The vaccine administered to induce or enhance immune
response to nonviral pathogens includes a stress protein
30   of the pathogen against which an immune response is
desired, a portion of that protein of sufficient size to

-20-

stimulate the desired immune response or a protein or
amino acid sequence (e.g., a polypeptide) which is the
functional equivalent of the pathogen stress protein in
that it is sufficiently homologous in sequence to that of
05 the stress protein to be capable of eliciting the desired
response. The term "sufficiently homologous in sequence
to that of the stress protein" means that the sequence of
the protein or polypeptide will generally show
approximately 50% identity with the stress protein amino
10 acid sequence. The vaccine can also include an adjuvant,
an appropriate carrier and an appropriate buffer. The
protein or amino acid sequence present in the vaccine can
be produced using known techniques. For example, it can
be obtained (isolated) from a source in which it occurs
15 in nature, can be produced by cloning and expressing the
gene encoding the desired stress protein or stress
protein portion or can be synthesized chemically or
mechanically.

In the case of a pathogen, such as a virus or a
20 transforming agent (i.e., an agent whose activity results
in production or formation of cancer cells), which does
not express its own stress proteins, the following
approach is used to enhance immune response or normal
immune surveillance (i.e., the ability of the immune
25 system to recognize self as well as foreign proteins).
A vaccine which includes a bacterial, mycobacterial or
other stress protein can be administered. Although there
are no viral stress proteins to be used for this purpose,
administration of such a vaccine will enhance the
30 existing immune surveillance system. In addition, immune
surveillance can also be enhanced by applying local heat

-21-

or any other substances or changes in condition which
induce the stress response in the individual being
treated. (This can also be employed in conjunction with
the vaccine, described previously, administered to
05 enhance immune response to a stress protein-producing
pathogen.) It is known that increased levels of stress
proteins are produced in many types of cancer cells.
Enhancement of the immune surveillance system, as
described, can be used to facilitate destruction and/or
10 to prevent progression or establishment of cancer cells.

      The method of the present invention can also be used
to modify or modulate an individual's response to his or
her own cells (e.g., as in autoimmune diseases). There
are at least two ways in which the present invention can
15 be used immunotherapeutically. First, stress proteins,
such as heat shock protein (hsp) 70 and hsp60, are known
to be involved in autoimmune disease. It is, thus,
possible to turn down an individual's response to "self"
by administering the appropriate stress protein(s) in
20 such a manner that the individual becomes more tolerant
of the protein. Second, because it is known that the
immune response in autoimmune diseases is to stress
proteins, it is possible to selectively inhibit or
interfere with the ability of immune cells which normally
25 interact with such proteins to do so. This can be done,
for example, by administering monoclonal antibodies that
bind to specific T cell receptors and delete or disable
such cells. Alternatively, rather than knocking out
immune cells, the stress response in all cells can be
30 turned down by administering a drug capable of reducing a
cell's ability to undergo the stress response. For

-22-

example, a drug targeted to or specific for heat shock
transcription factor, which is needed to stimulate heat
shock genes, can be administered. The transcription
factor is rendered nonfunctional or subfunctional and, as
05 a result, cells' ability to undergo the stress response
is also lessened.

The present invention is further illustrated by the
following exemplification, which is not intended to be
limiting in any way.

10 <u>EXEMPLIFICATION</u>

<u>Recombinant DNA Clones</u>. The isolation and
characterization of <u>M. tuberculosis</u> and <u>M. leprae</u> λgt11
genomic DNA clones with murine monoclonal antibodies have
been described. Husson, R.N. and Young, R.A., <u>Proc.</u>
15 <u>Natl. Acad. Sci., USA</u>, <u>84</u>:1679-1683 (1987); Young, R.A.,
<u>et al.</u>, <u>Nature</u>, (London) <u>316</u>:450-452 (1985). DNA was
isolated from these clones and was manipulated by
standard procedures. Davis, R.W., <u>Advanced Bacterial</u>
<u>Genetics</u>: A Manual for Genetic Engineering (Cold Spring
20 Harbor Lab., Cold Spring Harbor, NY), (1980).

<u>DNA Sequence Analysis</u>. DNA was subcloned into vector
M13mp18 or M13mp19 (New England Biolabs), as suggested by
the supplier. Dideoxynucleotide chain-termination
reactions and gel electrophoresis of the sequenced
25 produced were as described. Davis, R.W., <u>Advanced</u>

<u>Bacterial Genetics</u>: A Manual for Genetic Engineering
(Cold Spring Harbor Lab., Cold Spring Harbor, NY),

(1980). DNA sequences were determined for both strands
of DNA. Computer analysis of sequences with UWGCG
programs was as described by Devereux, J., et al. Nucleic
Acids Res., 12:387-395 (1984).

05   Immunoblot Analysis. Escherichia coil strain TG1 was
transformed with the following plasmids by standard
procedures (Maniatis, T., et al., Molecular Cloning, A
Laboratory Manual (Cold Spring Harbor Lab., Cold Spring
Harbor, NY) (1982), with selection for ampicllin
10   resistance: pND5, a derivatrive of pBR325 containing the
E. coli groE genes (Jenkins, A.J., et al., Mol. Gen.
Genet, 202:446-454 (1986); pUC8 (Vic?, J., Gene,
19:259-268 (1982); pUC8 with insert DNA for λgt11 clone
Y3178 (M. leprae 65-kDa antigen, Young, R.A., et al.,
15   Nature, (London) 316:450-452 (1985)) ligated in the EcoRI
site.
       Overnight cultures of E. coli strains in
Luria-Bertani (LB) medium were centrifuged and
resuspended in isotonic phosphate-buffered saline at a
20   cell density corresponding to an absorbance of 20 at 60
nm. An equal volume of sample buffer containing 2%
(wt/vol) polycrylamide gels in the presence of $NaDodSO_4$
was added, and, after heating on a boiling water bath for
2 min, 5-ml samples were electrophoresed on 12% (wt/vol)
25   polycrylamide gels in the presence of $NaDodSO_4$. Blots
were prepared by electrophoretic transfer of the proteins
to a nitrocellulose membrane, and binding of monoclonal
antibodies was assayed with a peroxidase-conjugated
secondary antibody as described. Young., D.B., et al.,
30   Infect. Immun., 55:1421-1425 (1987).

-24-

Six M. tuberculosis and six leprae proteins have
been implicated in the immune response to the
mycobacterial pathogens (T1). To obtain clues to the
normal cellular function of several of these
05 mycobacterial antigens, DNA clones encoding these
proteins, isolated by using monoclonal antibodies to
probe lambdagt11 libraries (Husson, R.N. and Young, R.A.,
Proc. Natl. Acad. Sci., USA, 84:1679-1683 (1987); Young,
R.A., et al., Nature, (London) 316:450-452 (1985)) were
10 subjected to sequence analysis. The sequences elucidated
have been submitted to the GenBank sequence database.
The Mycobacterial 71-k Da Antigen. The 71-k Da
antigen of M. tuberculosis is recognized by human T cells
during infection (Table).

-25-

TABLE   Mycobacterial protein antigens

Protein, kDA	Recognized by human T cells	Subjected to sequence analysis	Homology with known proteins
05 M. tuberculosis			
71	+	+	DnaK
65*	+	+	GroEL
38	+	-	-
19	+	+	None
10 14	+	-	
12	ND	-	-
M. leprae			
70	ND	-	DnaK
65	+	+	GroEL
15 36	+	-	-
28	+	-	-
18	+	+	Plant Hsp
12	ND	-	-

20   Mycobacterial protein antigens, their recognition by
human T cells, and homology of the deduced mycobacterial
protein sequences to known proteins are summarized.
ND, not determined; +, yes; -, no
*   Includes data derived from study of the 65-kDA
25 antigens of M. bovis BCG (bacillus Calmette-Guerin),
which is identical to the M. tuberculosis 65-kDA antigen.
+   A. S. Mustafa, J. R. Lamb, D. Young and R. A. Young,
unpublished data.

-26-

The insert DNA of lambdagt11 clone Y3271 (Husson, R.N. et al., Proc. Natl. Acad. Sci., USA, 84:1679-1683 (1987), was sequenced to obtain amino acid sequence information for the 71-kDa antigen of M. tuberculosis. This clone

05 produces a beta-galactosidase fusion protein containing the carboxyl-terminal one-third of the 71-kDa antigen exhibits 40% amino acid sequence identity with the comparable segment of the dnaK gene product from E coli (Bardwell, J.C., et al., Proc. Natl. Sci., USA,

10 81:848-852 (1984)), (Fig. 1). Fig 2A shows the extent of sequence similarity between portions of the mycobacterial and the E. coli 70-k Da polypeptides. Sequences transcriptionally downstream from the mycobacterial 71-k Da gene predict a 356-amino acid protein homologous to

15 the E. coli dnaJ gene product (unpublished data), indicating that the E. coli dnaK-dnaJ operon structure is conserved in M. tuberculosis and consistent with the conclusion that the mycobacterial 71-kDa antigen is a homologue of the E. coli dnaK gene product. The product

20 of the dnaK gene is a member of the 70-kDa heat shock protein family that is highly conserved among prokaryotes and eukaryotes (Bardwell, J.C., et al., Proc. Natl. Sci., USA, 81:848-852 (1984); Lindquist, S., Annu. Rev. Biochem., 55:1151-1191 (1986).

25   The M. leprae 70-k Da antigen cross-reacts with monoclonal antibodies directed to the M. tuberculosis and M. leprae are both members of the 70-k Da heat shock protein family of stress proteins.

-27-

   The Mycobacterial 65-kDa antigen. The 65-kDa
antigens of M. tuberculosis and M. leprae are involved in
in the human T-cell response to mycobacterial infection
(Table). Genes encoding these proteins have been
05 isolated (Husson, R.N. and Young, R.A., Proc. Natl. Acad.
Sci., USA, 84:1679-1683 (1987); Young, R.A., et al.,
Nature, (London) 316:450-452 (1985)) and sequenced
(Shinnick, T.M., J. Bacteriol., 169:1080-1088 (1987);
Mehram, V., et al., Proc. Natl. Acad. Sci., USA,
10 83:7013-7017 (1986)), revealing that the amino acid
sequences of the 65-kDa antigens of M. tuberculosis and
M. leprae are 95% identical. These protein sequences
exhibit no significant sequence similarity to proteins in
the GenBank database.
15   Identification of these proteins was based on the
observation that some monoclonal antibodies directed
against the mycobacterial 65-kDa antigens cross-react
with an E. coli protein of 60kDa. E. coli cells
transformed with the plasmid pND5 (Sanger, F., et al.,
20 Proc. Natl. Acad. Sci., USA, 74:5463-5467 (1977), which
contains the E. coli gro E genes, had been shown to
accumulate large amounts of the 60-kDa protein. A
comparison of the mycobacterial 65-kDa protein sequences
with those determined for E. coli groEL (C. Woolford, K.
25 Tilly, C. Georgopoulous, and R.H., unpublished data)
revealed the extent of the sequence similarity as shown
in Fig. 1B.
   The 60-kDa Gro EL protein is a major stress protein
in E. coli. Lindquist, S., Annual Rev. Biochem.,
30 55:1151-1191 (1986); Nature, 333:330-334 (1988). There
is some evidence that the mycobacterial 65-kDa proteins

-28-

accumulate in response to stress: Mycobacterium bovis
BCG (bacillus Calmette-Guerin) cultures grown in
zinc-deficient medium are substantially enriched in this
protein (De Bruyn, J., et al., Infect. Immun., 55:245-252
05 (1987)). This infers that the 65-kDa proteins of M.
tuberculosis and M. leprae are homologues of the E. coli
Gro EL protein.

Other Mycobacterial Antigens. T lymphocytes that
respond to the M. tuberculosis 19-kDa antigen and the M.
10 leprae 18-kDa antigen have been observed in humans with
tuberculosis and leprosy, respectively (Table 1). DNA
encoding these antigens was sequenced from the Agtll
clones Y3148 (Husson, R.N. and Young, R.A., Proc. Natl.
Acad. Sci., USA, 84:1679-1683 (1987); and Y3179 (Young,
15 R.A., et al., Nature, (London) 316:450-452 (1985)),
respectively. The M. tuberculosis 19-kDa protein
sequence predicted from the DNA exhibited no significant
sequence similarity to proteins in the GenBank database.
However, the M. leprae 18-kDa protein sequence was
20 similar to the soybean 17-kDa heat shock protein, a
protein representation of a major class of plant heat
shock proteins (Schoffl, F. and Van Bogelen, R.A., In:
Escherichia coli and Salmonella typhimurium, Cellular and
Molecular Biology, Am. Soc. Microbiol., Washington, D.C.
25 (1987).

Equivalents
Those skilled in the art will recognize, or be able
to ascertain using no more than routine experimentation,
many equivalents to the specific embodiments of the
30 invention described specifically herein. Such
equivalents are intended to be encompassed in the scope
of the following claims.

-29-

CLAIMS

1.   A vaccine comprising all or a portion of a selected
        stress protein or all or a portion of a protein
        having an amino acid sequence sufficiently
05   homologous to the amino acid sequence of the stress
        protein.

2.   A vaccine of Claim 1 in which the stress protein is
        a mycobacterial stress protein or a protein having
        an amino acid sequence sufficiently homologous to
10   the amino acid sequence of the mycobacterial stress
        protein.

3.   A composition for use as an agent to induce immune
        tolerance, comprising a selected stress protein.

4.   A composition for use in treating an autoimmune
15   disease, comprising all or a portion of a selected
        stress protein or all or a portion of a protein
        having an amino acid sequence sufficiently
        homologous to the amino acid sequence of the stress
        protein.

20 5.   A composition of Claim 4 for treating rheumatoid
        arthritis.

6.   A vaccine for use in enhancing in an individual the
        immune response to a nonviral pathogen, comprising
        all or a portion of a stress protein of the nonviral
25   pathogen against which the enhanced response is
        desired.

-30-

7.   A vaccine of Claim 6 in which the stress protein is
     a mycobacterial stress protein.

8.   A method of producing or enhancing an immune
     response in an animal, comprising administering to
05   the animal a selected stress protein, in sufficient
     quantity to elicit the desired immune response.

9.   A method of immunizing an animal against a
     subsequent nonviral infection, comprising
     administering to the animal a selected stress
10   protein, in sufficient quantity to produce an immune
     response.

10. A method of inducing in an individual immune
     tolerance against a protein, comprising
     administering to the individual all or a portion of
15   a selected stress protein or all or a portion of a
     protein having an amino acid sequence sufficiently
     homologous to the amino acid sequence of the stress
     protein, under conditions appropriate for induction
     of the desired tolerance.

20 11. A method of Claim 10, wherein the protein is a
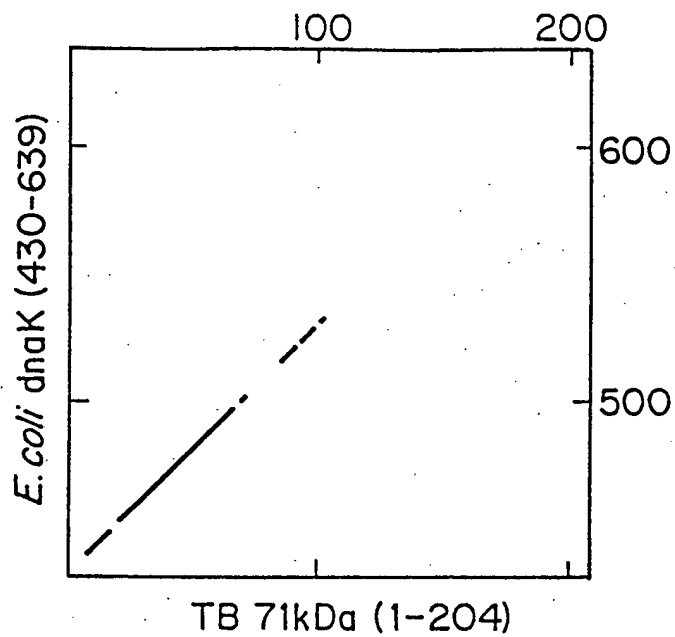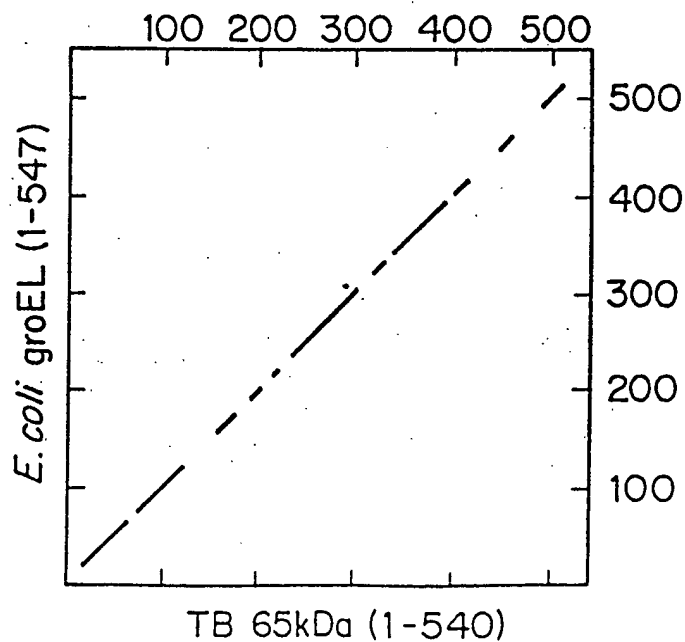   protein associated with rheumatoid arthritis.

Fig. 1A

Fig. 1B

FIGURE 2

```
             10        20        30        40        50        60        70
             ,         ,         ,         ,         ,         ,         ,
HUMP1    1   MLRLPTVFRQMRPVSRVLAPHLTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGRTVIIEQSWGS
                                       :   :::   :::::   :: :::::: : ::::::: :
GROEL        MA------AKDVKFGNDARVKMLRGVNVLADAVKVTLGPKGRNVVLDKSFGA

             80        90        100       110       120       130       140
             ,         ,         ,         ,         ,         ,         ,
HUMP1   71   PKVTKDGVTVAKSIDLKDKYKNIGAKLVQDVANNTNEEAGDGTTTATVLARSIAKEGFEKISKGANPVEI
             : ::::: ::  :  :  : ::  : :   :: :::::::::::::: :   :: ::
GROEL        PTITKDGVSVAREIEPEDKFENMGAQMVKEVASKANDAAGDGTTTATVLAQAIITEGLKAVAAGMNPMDL

             150       160       170       180       190       200       210
             ,         ,         ,         ,         ,         ,         ,
HUMP1  141   RRGVMLAVDAVIAELKKQSKPVTTPEEIAQVATISANGDKEIGNIISDAMKKVGRKGVITVKDGKTLNDE
             :  : ::  : ::  : :     :::::::: ::::: ::  ::    : ::::::: :::::  :  ::
GROEL        KRGIDKAVTAAVEELKALSVPCSDSKAIAQVGTISANSDETVGKLIAEAMDKVGKEGVITVEDGTGLQDE

             220       230       240       250       260       270       280
             ,         ,         ,         ,         ,         ,         ,
HUMP1  211   LEIIEGMKFDRGYISPYFINTSKGQKCEFQDAYVLLSEKKISSIQSIVPALEIANAHRKPLVIIAEDVDG
             : ::::: ::::: ::::::: :      :  :: :::: ::  : :    :::  :: :: ::::::::
GROEL        LDVVEGMQFDRGYLSPYFINKPETGAVELESPFILLADKKISNIREMLPVLEAVAKAGKPLLIIAEDVEG

             290       300       310       320       330       340       350
             ,         ,         ,         ,         ,         ,         ,
HUMP1  281   EALSTLVLNRLKVGLQVVAVKAPGFGDNRKNQLKDMAIATGGAVFGEEGLTLNLEDVQPHDLGKVGEVIV
             :::  : : :  : :  ::::::::::  :  : :::  :: ::: ::  :   ::     ::: :
GROEL        EALATAVVNTIRGIVKVAAVKAPGFGDRRKAMLQDIATLTGGTVISEE-IGMELEKATLEDLGQAKRVVI

             360       370       380       390       400       410       420
             ,         ,         ,         ,         ,         ,         ,
HUMP1  351   TKDDAMLLKGKGDKAQIEKRIQEIIEQLDVTTSEYEKEKLNERLAKLSDGVAVLKVGGTSDVEVNEKKDR
             ::  :  :      : :   :  : ::  :::::::::   ::: :::::  :::  ::   : : :::
GROEL        NKDTTTIIDGVGEEAAIQGRVAQIRQQIEEATSDYDREKLQERVAKLAGGVAVIKVGAATEVEMKEKKAR
```

FIGURE 2 CONT'D

```
        421    430       440        450         460        470        480        490
             ,      ,          ,           ,           ,          ,          ,          ,
HUMP1   VTDALNATRAAVEEGIVLGGGCALLRCIPALDSLTPANEDQKIGIEIIKRTLKIPAMTIAKNAGVEGSLI
             ::  ::: :::::::::  :: :     ::  :: :::::  ° ° ° ° °:
GROEL   VEDALHATRAAVEEGVVAGGGVALIRVASKLADLRGQNEDQNVVSSSL-RAMEAPLRQIVLNCGEEPSVV

        491    500       510        520         530        540        550        560
             ,      ,          ,           ,           ,          ,          ,          ,
HUMP1   VEKIMQSSSEVGYDAMAGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEVVVTEIPKEEKDPGMGA
             ::  :   ::: ::::: :::  :::::::::: :::: :: ::: :  :: ::   :: :::::
GROEL   ANTVKGGDGNYGYNAATEEYGNMIDMGILDPTKVTRSALQYAASVAGLMITTECMVTDLPKND-AADLGA

        561    570
             ,      ,
HUMP1   MGGMGG--GMGGGMF
             :::::: :::::: :
GROEL   AGGMGGMGGGMGGMM-
```

Total score = 4667, 5 breaks
  276 identities out of 545 possible matches between residues

25 random runs
Alignment score =   65.34 SD   Standard deviation =   18.94   Mean = 3429.48

FIGURE 3

```
              1,        10,        20,        30,        40,        50,        60,        70,
HUMP1    MLRLPTVFRQMRPVSRVLAPHLTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGRTVIIEQSWGS
           :                                  ::    ::  :::::: :  ::::: :  :::
ML65K    M-----------AKTIAYDEEARRGLERGLNSLADAVKVTLGPKGRNVVLEKKWGA

              71,       80,        90,        100,       110,       120,       130,       140,
HUMP1    PKVTKDGVTVAKSIDLKDKYKNIGAKLVQDVANNTNEEAGDGTTTATVLARSIAKEGFEKISKGANPVEI
           : : ::: :: ::  ::::  ::   :::::::::::::::  :::          :::
ML65K    PTITNDGVSIAKEIELEDPYEKIGAELVKEVAKKTDDVAGDGTTTATVLAQALVKEGLRNVAAGANPLGL

              141,      150,       160,       170,       180,       190,       200,       210,
HUMP1    RRGVMLAVDAVIAELKKQSKPVTTPEEIAQVATISANGDKEIGNIISDAMKKVGRKGVITVKDGKTLNDE
           :::   : : :: ::::  :: :  : : ::  ::  ::  ::::  :  :::
ML65K    KRGIEKAVDKVTETLLKDAKEVETKEQIAATAAISA-GDQSIGDLIAEAMDKVGNEGVITVEESNTFGLQ

              211,      220,       230,       240,       250,       260,       270,       280,
HUMP1    LEIIEGMKFDRGYISPYFINTSKGQKCEFQDAYVLLSEKKISSIQSIVPALEIANAHRKPLVIIAEDVDG
           :: ::::::: : :::::: :::        :  :       :  :: :  ::::::: :
ML65K    LELTEGMRFDKGYISGYFVTDAERQEAVLEEPYILLVSSKVSTVKDLLPLLEKVIQAGKSLLIIAEDVEG

              281,      290,       300,       310,       320,       330,       340,       350,
HUMP1    EALSTLVLNRLKVGLQVVAVKAPGFGDNRKNQLKDMAIATGGAVFGEEGLTNLEDVQPHDLGKVGEVIV
           :::::::: : :::::::::: ::::::::  :: : :::::  :::  :  :::       :::  :
ML65K    EALSTLVVNKIRGTFKSVAVKAPGFGDRRKAMLQDMAILTGAQVISEE-VGLTLENTDLSLLGKARKVM

              351,      360,       370,       380,       390,       400,       410,       420,
HUMP1    TKDDAMLLKGKGDKAQIEKRIQEIIEQLDVTTSEYEKEKLNERLAKLSDGVAVLKVGGTSDVEVNEKKDR
           :::  : :    :   :   :  :: : :       :::::::: ::  ::  ::  :   ::  :
ML65K    TKDETTIVEGAGDTDAIAGRVAQIRTEIENSDSDYDREKLQERLAKLAGGVAVIKAGAATEVELKERKHR
```

FIGURE 3 CONT'D

```
                421        430        440        450        460        470        480        490
HUMP1           VTDALNATRAAVEEGIVLGGGCALLRCIPALDSLTPANEDQKIGIEIIKRTLKIPAMTIAKNAGVEGSLI
                ::       ::::::::: :::  ::   ::::: :       ::  :  ::  ::  ::  ::  ::
ML65K           IEDAVRNAKAAVEEGIVAGGGVTLLQAAPALDKLKLTGDEAT-GANIVKVALEAPLKQIAFNSGMEPGVV

                491        500        510        520        530        540        550        560
HUMP1           VEKIMQSSSEVGYDAMAGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEVVVTEIPKEEKDPGMGA
                ::  ::   ::  ::  : ::  ::  ::  :: ::: :  ::  ::  :: : ::  :::  :
ML65K           AEKVRNLSVGHGLNAATGEYEDLLKAGVADPVKVTRSALQNAASIAGLFTT-EAVVADKPEKTAAPASDP

                561        570
HUMP1           MGGMGGGMGGGMF
                ::::::: :  ::
ML65K           TGGMGG-MD---F

Total score = 4552, 7 breaks
255 identities out of 540 possible matches between residues

25 random runs
Alignment score =     47.73 SD     Standard deviation =     23.86     Mean = 3413.16
```

FIGURE 4

```
            1          10         20         30         40         50         60         70
HUMP1     MLRLPTVFRQMRPVSRVLAPHLTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGRTVIIEQSWGS
                                                          :  :::::: ::  ::::: :: ::
TB65K     M-----------------------------------AKTIAYDEEARRGLERGLNALADAVKVTLGPKGRNVVLEKKWGA

            71         80         90        100        110        120        130        140
HUMP1     PKVTKDGVTVAKSIDLKDKYKNIGAKLVQDVANNTNEEAGDGTTTATVLARSIAKEGFEKISKGANPVEI
            :  : ::: :  :::::: ::  :  ::::::::::: :::: ::::  :::   :::
TB65K     PTITNDGVSIAKEIELEDPYEKIGAELVKEVAKKTDDVAGDGTTTATVLAQALRKEGLRNVAAGANPLGL

            141        150        160        170        180        190        200        210
HUMP1     RRGVMLAVDAVIAELKKQSKPVTTPEEIAQVATISANGDKEIGNIISDAMKKVGRKGVITVKDGKTLNDE
            ::    :  :  :::: ::   :::: ::::: ::  :::::::::    :::   :::
TB65K     KRGIEKAVEKVTETLLKGAKEVETKEQIAATAAISA-GDQSIGDLIAEAMDKVGNEGVITVEESNTFGLQ

            211        220        230        240        250        260        270        280
HUMP1     LEIIEGMKFDRGYISPYFINTSKGQKCEFQDAYVLLSEKKISSIQSIVPALEIANAHRKPLVIIAEDVDG
            ::::::::::::::::: ::  : ::  :: :: ::::::::: :::::::::
TB65K     LELTEGMRFDKGYISGYFVTDPERQEAVLEDPYILLVSSKVSTVKDLLPLLEKVIGAGKPLLIIAEDVEG

            281        290        300        310        320        330        340        350
HUMP1     EALSTLVNRLKVGLQVVAVKAPGFGDNRKNQLKDMAIATGGAVFGEEGLTNLEDVQPHDLGKVGEVIV
            :::::::::: :  :::::::::::::::: :: ::: :: :::  :::  :::   :: ::
TB65K     EALSTLVVNKIRGTFKSVAVKAPGFGDRRKAMLQDMAILTGGQVISEE-VGLTLENADLSLLGKARKVVV

            351        360        370        380        390        400        410        420
HUMP1     TKDDAMLLKGKGDKAQIEKRIQEIIEQLDVTTSEYEKEKLNERLAKLSDGVAVLKVGGTSDVEVNEKKDR
            :::   :: :  :: ::::   :::  :  :::::: :::::::::   :: :::::::
TB65K     TKDETTIVEGAGDTDAIAGRVAQIRQEIENSDSDYDREKLQERLAKLAGGVAVIKAGAATEVELKERKHR
```

FIGURE 4 CONT'D

```
           421         430         440         450         460         470         480         490
HUMP1      VTDALNATRAAVEEGIVLGGGCALLRCIPALDSLTPANEDQKIGIEIIKRTLKIPAMTIAKNAGVEGSLI
             ::     ::::::::::: :::  ::     :   :      :      ::  :  :   :  ::  ::
TB65K      IEDAVRNAKAAVEEGIVAGGGVTLLQAAPTLDELK-LEGDEATGANIVKVALEAPLKQIAFNSGLEPGVV

           491         500         510         520         530         540         550         560
HUMP1      VEKIMQSSSEVGYDAMAGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAEVVVTEIPKEEKDPGMGA
             ::     :   :   :         :: :: :: :: :  :  ::  :          ::  ::   ::
TB65K      AEKVRNLPAGHGLNAQTGVYEDLLAAGVADPVKVTRSALQNAASIAGLFLTTEAVVADKPEKEKASVPG-

           561         570
HUMP1      MGGMGGGMGGGMF
             :: : ::: :
TB65K      -----GGDMGGMDF
```

Total score = 4560, 5 breaks
257 identities out of 540 possible matches between residues

25 random runs
Alignment score = 49.36 SD   Standard deviation = 23.23   Mean = 3413.16

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATI N OF SUBJECT MATTER (if several classification symbols apply, indicate all) [o]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: A 61 K 37/02, A 61 K 39/04

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N, A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [o]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [o]

Category [o]	Citation of Document, [11] with Indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0262170 (DE STAAT DER NEDERLANDEN) – 6 April 1988, see the whole document	1-5
X	WO, A, 88/00974 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 11 February 1988, see claims 8-17	1,2,6,7
X	Proc. Natl. Acad. Sci, USA, vol. 85, June 1988, (US) D. Young et al.: "Stress proteins are immune targets in leprosy and tuberculosis", pages 4267-4270, see the whole article	1-7
X	Proc. Natl. Acad. Sci, USA, vol. 84, March 1987 (US) R.N. Husson et al.: "Genes for the major protein antigens of Mycobacterium tuberculosis: The etiologic agents of tuberculosis and leprosy share an immunodominant antigen",	1,2,6,7

[o] Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
8th September 1989	26. 10. 89

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	T K WILLIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	pages 1679-1683, see the whole article	
X	Nature, vol. 316, 1 August 1985, R.A. Young et al.: "Genes for the major protein antigens of the leprosy parasite Mycobacterium leprae", pages 450-452, see the whole article	1,2,6,7
X	Infection & Immunity, vol. 55, no. 6, 1987, American Soc. for Microbiology (US) J.E.R. Thole et al.: "Characterization, sequence determination, and immunogenicity of a 64-kilodalton protein of Mycobacterium bovis BCG expressed in Escherichia coli K-12", pages 1466-1475, see the whole article	1,2,6,7
A	WO, A, 85/05034 (UNIVERSITY COLLEGE, LONDON) 21 November 1985, see claims	1-5
P,X	WO, A, 88/05823 (WHITEHEAD INSTITUTE FOR MEDICAL RESEARCH) 11 August 1988, see the whole document	1,2,6,7
P,X	WO, A, 88/06591 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 7 September 1988, see claims	1,2,6,7

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☑ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☒ Claim numbers 8-11 because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(IV)
Methods for treatment of the human or animal body by
surgery or therapy, as well as diagnostic methods.

2.☐ Claim numbers............, because they relate to parts of the International application that do not comply with the prescribed require-
ments to such an extent that no meaningful International search can be carried out, specifically:

3.☐ Claim numbers............, because they are dependent claims and are not drafted in accordance with the second and third sentences of
PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple Inventions in this International application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims
of the International application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only
those claims of the International application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to
the Invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not
invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 18/10/89
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0262170	06-04-88	FR-A-	2596593	02-10-87
		WO-A-	8706073	08-10-87
		JP-T-	63502871	20-10-88
		US-A-	4821004	11-04-89
WO-A- 8800974	11-02-88	AU-A-	7801387	24-02-88
		EP-A-	0316354	24-05-89
WO-A- 8505034	21-11-85	AU-A-	4297685	28-11-85
		EP-A-	0181364	21-05-86
		JP-T-	61502258	09-10-86
		US-A-	4716038	29-12-87
WO-A- 8805823	11-08-88	AU-A-	1548388	24-08-88
WO-A- 8806591	07-09-88	AU-A-	1495688	26-09-88
		EP-A-	0305488	08-03-89
		ZA-A-	8801344	26-08-88